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Ethylene Interacts with Auxin in Regulating Developmental Attenuation of Gravitropism in Flax Root

Zhong Ma · Yu-yu Ren

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Abstract Previous research shows that gravity-sensing in flax (*Linum usitatissimum*) root is initiated during seed imbibition and precedes root emergence. In this study we investigated the developmental attenuation of flax root gravitropism post-germination and the involvement of ethylene. Gravity response deteriorated significantly from 3 to 11 h after root emergence, which occurred at around 19 h after imbibition (that is, from "age" 22 to 30 h). Although the root elongation rate increased from 22 to 30 h, the gravitropic curving rate declined steadily. Older roots were able to tolerate higher levels of exogenous IAA before inhibition of elongation and gravitropism occurred. The age-dependent effect of IAA on root growth and gravitropism suggests that young roots are more sensitive to auxin and respond to a smaller vertical auxin gradient than older roots upon horizontal gravistimulation. The ethylene synthesis inhibitor AVG (2-aminoethoxyvinyl glycine, 10 μM) or ethylene action inhibitor Ag^+ (10 μM) stimulated gravitropic curvature of 30 h roots by 24 and 32%, respectively, but had no effect on 22 h roots, suggesting that as roots age, ethylene begins to play a role in root gravitropism. The auxin transport inhibitor NPA (*N*-naphthylphthalamic acid, 50 μM) reduced gravitropic curvature of 30 h roots by 24% but had no effect on 22 h roots. On the other hand, treating roots simultaneously with the auxin transport inhibitor and ethylene synthesis or action inhibitor stimulated gravitropic curvature of 30 h roots but not 22 h roots. Taken together, these data indicate that as roots develop, their weakened gravity response is due to decreased auxin sensitivity and possibly auxin transport regulated by ethylene.

Keywords Gravitropism · Developmental attenuation · Auxin · Ethylene · Flax roots

Introduction

The ability of plant roots to respond to and grow in the direction of gravity is critical in the early development of seedlings, as root gravitropism determines the orientation of an emerging root and the subsequent anchorage of the plant in the soil. Newly emerged seedling roots are especially sensitive to gravity, which provides a directional cue that leads root growth toward the belowground sources of water and mineral nutrients (Muday and Rahman 2007). Proper orientation of seedling roots can be fulfilled best if gravity is sensed prior to root emergence. Indeed, in flax embryonic roots, the inception of gravisensitivity is established during seed imbibition, well in advance of germination (Ma and Hasenstein 2006). Although most studies on root gravitropism have been conducted on emerged primary roots of young seedlings at a particular length or age, temporal changes in root gravitropism as a root develops have received very little attention. How primary root gravitropism might change over time with the development of more complex root architecture (for example, formation of lateral root branching), and the mechanisms involved remain interesting and largely unanswered questions. Knowledge on the temporal dynamics of gravitropism would provide us with a better understanding of the gravitropic behavior of the entire root system as it develops.

Z. Ma (✉) · Y. Ren
Department of Biology, Truman State University,
100 East Normal Street, Kirksville, MO 63501, USA
e-mail: johnma@truman.edu

Y. Ren
Program in Biomedical Sciences, University of Michigan,
Ann Arbor, MI 48109, USA

In roots of higher plants, gravity-sensing occurs in specialized sensory cells in the root cap, where physical information about root orientation with respect to the gravity vector is perceived (Blancaflor and others 1998; Juniper and others 1966; Sack 1991; Volkmann and Sievers 1979). A change in root axis alignment with gravity triggers a cascade of signal transduction events in the sensing cells; a biochemical signal is then transmitted to the elongation zone, which in turn exhibits the characteristic bending growth toward gravity due to differential cell elongation (Björkman 1988; Kiss 2000; Sack 1997). The differential growth displayed during the gravitropic response has been linked to a lateral auxin gradient across the root (Chen and others 2002; Friml 2003; Muday 2001), and several auxin transport protein carriers have been identified (Chen and others 1998; Friml 2003; Swarup and others 2001). A lateral auxin gradient is formed in gravistimulated *Arabidopsis* roots as revealed by auxin-responsive reporter genes (Boonsirichai and others 2003; Ottenschlaeger and others 2003; Rashotte and others 2001). PIN3, a putative auxin efflux carrier, is uniformly localized around the columella cells in vertically placed roots but is preferentially relocated to the lower side of the cell when the root is reoriented horizontally; this relocation is thought to direct auxin flow from the root cap to the elongation zone (Friml and others 2002).

In addition to auxin, ethylene is yet another plant hormone that has been shown to regulate gravitropism. Ethylene has multiple developmental and physiological effects on plant growth, development, and stress responses, and its biosynthesis is affected by factors such as developmental stage, environment, auxin, and physical stress (for review, see Alonso and Stepanova 2004; Chen and others 2005; Lin and others 2009). In *Arabidopsis* roots, crosstalk between ethylene and auxin regulates root growth as well as root branching. Ethylene stimulates biosynthesis and basipetal transport of auxin, leading to inhibited cell growth in the root elongation zone (Růžička and others 2007; Swarup and others 2007); similarly, ethylene-stimulated auxin biosynthesis affects lateral root initiation depending on ethylene doses (Ivanchenko and others 2008; Negi and others 2008). In assessing its role in shoot and root gravitropism, ethylene gas and/or its precursor 1-aminocyclopropane carboxylic acid (ACC) has been used in a number of species with inconsistent results. Ethylene treatment has been shown to reduce or delay the gravitropic response of shoots of cocklebur, tomato, and *Arabidopsis* (Kiss and others 1999; Madlung and others 1999; Wheeler and others 1986), delay but enhance the final root gravitropic curvature of maize (Lee and others 1990), increase the rate of gravitropic curvature of maize roots (Chang and others 2004), and negatively regulate root gravitropism in *Arabidopsis* (Buer and others 2006). The exact mechanism of ethylene modulation of gravitropism is unclear. Ethylene was reported to reduce polar IAA transport in shoot and root tissues (Morgan and Gausman 1966; Prayitno and others 2006; Suttle 1988), as well as the lateral redistribution of auxin across gravistimulated shoots (Burg and Burg 1966) and corn roots (Lee and others 1990). In *Arabidopsis* root tips, elevated ethylene reduced root gravitropic curvature and induced accumulation of flavonoid, an endogenous auxin transport regulator (Buer and others 2006). Ethylene may also positively regulate auxin synthesis in the root tip (Stepanova and others 2005, 2007). These studies suggest that ethylene could regulate gravitropism by altering the transport and/or synthesis of auxin.

In this study we attempted to investigate the temporal attenuation of gravitropism in flax root by examining how auxin affects root growth and the graviresponse over time and the role of ethylene and how it might be involved in the action of auxin. Root gravitropism begins with gravity-sensing and signaling, a process that likely involves interactions of a series of molecules such as auxin and ethylene, and a number of cellular structures such as statoliths, endoplasmic reticulum (ER), vacuoles, and possibly the actin cytoskeleton (Sack 1997; Zheng and Staehelin 2001). Identifying developmentally related changes in gravitropism as roots age and the molecular and cellular basis for such changes is likely to help us uncover important clues about the development and temporal dynamics of root gravity-sensing machinery and further our understanding of gravity-related whole-root-system behavior and physiology.

Materials and Methods

Plant Material

Flax (*Linum usitatissimum*) seeds were soaked in distilled water for 10 min and then placed on strips of germination paper, with micropyles pointing in the same direction. The paper strips were allowed to dry completely so that seeds adhered to the paper by the mucilage produced during soaking. Two to three pieces of seeded paper strips were layered in parallel onto the surface of 20 ml of 1% (w/v) solidified phytagel medium in each plastic square petri dish (90 × 90 mm), resulting in a total of about 20–30 seeds per dish. The phytagel plate was prepared in buffer solution consisting of 1 mM KH_2PO_4 and K_2HPO_4 diluted from 100 mM stock solutions, and the pH was adjusted to 6.5. The petri dishes were wrapped with Parafilm and kept vertical for root emergence along the surface of the phytagel. Seeds were germinated in the dark at 22–23°C in a growth chamber, and root emergence generally occurred by 19 h after imbibition. Seedlings at 22, 26, and 30 h after seed imbibition, with an average root length (±SE,

$n = 20$–30) of 2.4 ± 0.2 mm at 22 h and 6.9 ± 0.4 mm at 30 h, were used in the experiments. For root-growth experiments, petri dishes were kept vertical so that the root axis was maintained in parallel to the direction of gravity. For gravitropic response experiments, petri dishes were rotated 90° sideways so that the long axes of the newly emerged roots were kept in a horizontal orientation. The growth and gravitropic curvature that developed over time were captured through time-lapse images using a digital camera. For quantification of growth and gravitropic curvature, Image-J software (ImageJ 1.42q, http://rsbweb.nih.gov/ij/) was used.

Temporal Changes in Gravitropism

The imbibition of three batches of flax seeds was initiated at three different time points, separated by 4 h time intervals, so that upon germination seedlings were at 22, 26, or 30 h after seed imbibition. Seedling roots were subsequently gravistimulated horizontally in square petri dishes turned 90° on their side and imaged every hour for 7 h. Root curvature was measured thereafter from time-lapse images.

Effect of Auxin, Ethylene Precursor ACC, and Inhibitors

To examine the effect of auxin or ethylene on root growth and gravitropic response, we incubated seedling roots of 22, 26, or 30 h in situ with IAA or ACC by carefully lowering the dishes vertically into 500 ml beakers containing IAA solutions, of concentrations ranging from 0.1 to 1,000 nM, or ACC (1-aminocyclopropane-1-carboxylic acid) solutions, of concentrations of 0.01, 1, or 100 μM. The lids of the petri dishes were removed and placed against the bottom of the petri dishes to maintain their vertical orientation and the roots were soaked in the solution for 20 min. After that, the plates were lifted from the solution and either kept in the same vertical orientation for time-lapse imaging of root growth or turned 90° on their side for horizontal gravistimulation and time-lapse imaging of gravitropic curvature development. In experiments examining the ethylene effect on gravitropism, ACC stock solution was diluted directly into warm phytagel made from buffered solution, in a concentration range similar to that used in the liquid solution.

In separate experiments, the auxin transport inhibitor *N*-naphthylphthalamic acid (NPA), the ethylene synthesis inhibitor 2-aminoethoxyvinyl glycine (AVG), and its action inhibitor Ag^+ were employed. In these experiments, all inhibitors were diluted directly into warm phytagel made from the buffered solution (pH 6.5); the concentrations of NPA used were 1, 10, and 50 μM, and the concentrations of AVG and Ag^+ used were 0.1, 1, and 10 μM.

Statistical Analysis

Statistical analyses of data were performed using Microsoft Excel software. p values reflect those of two-sided Student's t test analyses.

Results

Root Gravitropic Response Deteriorates Over Time After Emergence

We observed in flax that the strongest gravitropic curvature occurred immediately upon root emergence at the time of germination, or approximately 19 h after imbibition, with seedling roots pointing straight down in horizontally placed seeds; subsequently, the root gravitropic response deteriorated rapidly. Two hours after horizontal gravistimulation, root curvature began to differ significantly between roots of different ages ($p < 0.01$), with younger roots attaining greater curvature (that is, 22 > 26 > 30 h). Upon 3 h of horizontal gravistimulation, roots that were approximately 3 h after emergence (that is, 22 h roots) developed curvature 1.5 times that of roots that were 7 h after emergence (that is, 26 h roots, $p < 0.00001$), but nearly three times that of roots that were 11 h after emergence (that is, 30 h roots, $p < 0.00001$) (Fig. 1).

As Roots Age, Root Elongation Accelerates but Rate of Gravitropic Curving Declines Continuously

As roots aged from 22 to 30 h, the vertical elongation rate of 26- and 30-h roots increased by 32% ($p = 0.00001$) and 15% ($p = 0.004$; Fig. 2), respectively. On the other hand, the curving rate for horizontally gravistimulated roots decreased continuously with root aging; compared to 22 h roots, the root curving rate of 26- and 30-h roots decreased by 44% ($p < 0.00001$) and 60% ($p < 0.00001$; Fig. 2), respectively, during the 4 h of gravistimulation. The overall increase in vertical elongation rate and the corresponding decrease in the gravitropic curving rate with root aging suggest that these two processes are independent of each other.

Auxin Sensitivity Decreases with Root Aging

Exogenously applied IAA over the range of 0.1–1,000 nM largely inhibited elongation of very young (that is, 22 h) roots; however, growth of 26- and 30-h roots was stimu-

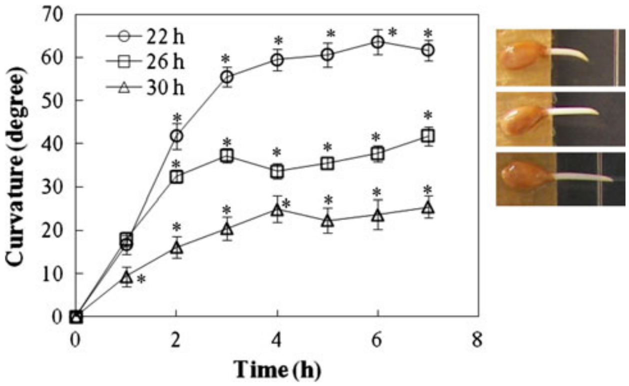

Fig. 1 Time course of gravitropic curvature development in horizontally oriented roots of three different ages, that is, 22, 26, or 30 h, counted from the beginning of imbibition. Seeds were imbibed in water and germinated on 1% phytagel plate. The average time for root emergence was at around 19 h after imbibition. Root curvature was measured every hour for 7 h following a 90° horizontal reorientation from the vertical direction. Each data point represents a mean ± SE of 54–90 roots from three replicates. Root curvature began to differ significantly between different ages 2 h after horizontal gravistimulation ($*p < 0.01$ by Student's t test; picture shows a representative root of each age at 2 h of horizontal gravistimulation)

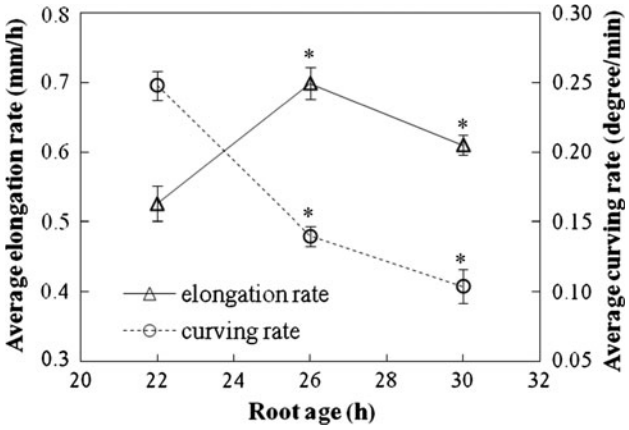

Fig. 2 Average elongation rates of vertically placed roots, and average curving rates of horizontally oriented roots of three different ages, that is, 22, 26, or 30 h, over a time course of 4 h. Average elongation or curving rates were calculated by dividing the change in length or curvature by 4 h, the time period during which growth or curvature occurred. Each data point represents a mean ± SE of 45–69 roots from three replicates. Statistical differences compared to 22 h roots were determined by Student's t test; $*p < 0.01$

lated by IAA at concentrations less than 10 and 100 nM, respectively, whereas growth inhibition occurred only beyond these concentrations for 26- and 30-h roots (Fig. 3a), suggesting increased tolerance (that is, decreased sensitivity) to IAA as roots age. The estimated IAA concentration that inhibits growth is close to 100 times greater for 26- than for 22-h roots, and nearly 600 times greater for 30- than for 22-h roots.

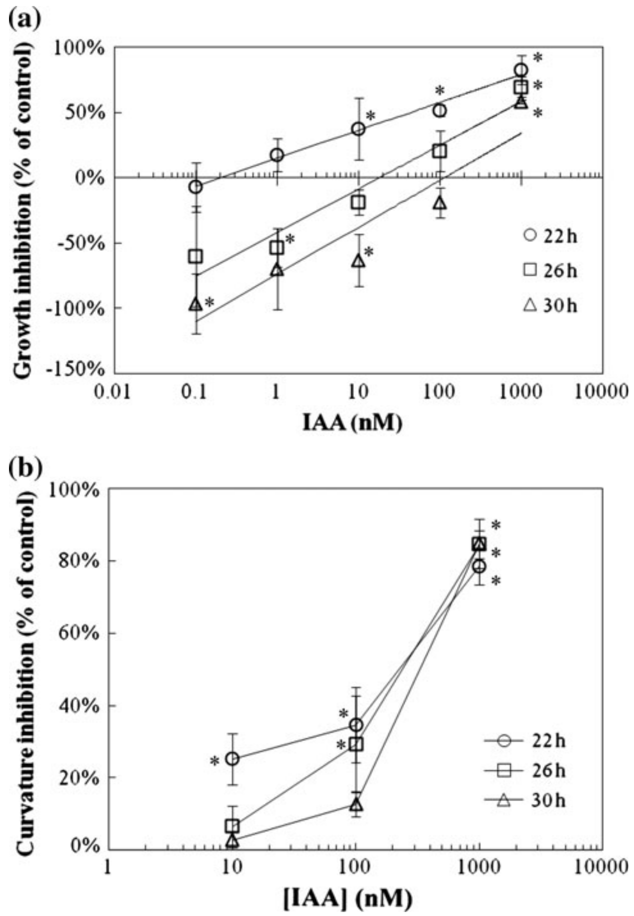

Fig. 3 The effect of exogenous auxin on **a** elongation and **b** gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. **a** Growth inhibition was calculated as the percentage reduction of growth rate compared to that of the control (that is, without auxin treatment) in each age group. Negative values in inhibition mean stimulation. The relationships between growth inhibition (y) and IAA concentration (x) are $y = 0.093\,\mathrm{Ln}(x) + 0.79$ for 22 h roots ($R^2 = 0.99$); $y = 0.15\,\mathrm{Ln}(x) + 0.58$ for 26 h roots ($R^2 = 0.95$); and $y = 0.16\,\mathrm{Ln}(x) + 0.34$ for 30 h roots ($R^2 = 0.88$). Each data point represents a mean ± SE of 18–36 roots from three replicates. **b** Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature inhibition was calculated as the percentage reduction in curvature compared to that of the control (that is, without auxin treatment) in each age group. Each data point represents a mean ± SE of 24–30 roots from three replicates. In both **a** and **b**, statistical differences compared to the untreated roots at each age were determined by Student's t test; $*p \leq 0.01$

Tissue Sensitivity to Auxin Gradient for Establishing Gravitropism Decreases as Roots Age

When horizontally gravistimulated roots are incubated in auxin solution, the natural auxin gradient across the top and bottom flanks of the roots is disrupted; therefore, treating roots with exogenous IAA decreases root gravitropic curvature. At a lower IAA concentration of 10 nM, the gravitropic curvature of 22 h roots was significantly inhibited compared to that of the control roots ($p = 0.01$;

Fig. 3b), whereas the curvature of 26- and 30-h roots was not affected; as IAA concentration increased from 10 to 1,000 nM, curvature inhibition intensified with the age of roots: inhibition of 30 h root gravitropic curvature increased by 30 times ($p < 0.00001$; Fig. 3b), whereas inhibition of 22 h root gravitropic curvature increased by only two times ($p < 0.00001$; Fig. 3b). This suggests that sensitivity to the IAA gradient across horizontally gravistimulated roots differs as roots age. To produce a gravitropic response, younger roots appear to require a smaller IAA gradient, which would be more sensitive to disruption by low external IAA concentrations.

ACC Reduces Elongation but Does Not Affect Gravitropic Response

The ethylene synthesis precursor ACC was used to examine the ethylene effect on root growth and gravitropic response. At low concentrations of ACC, that is, 0.01 and 1 μM, elongation of 22- and 30-h roots was not significantly affected compared to that of the control roots without ACC treatment; at an elevated ACC concentration of 100 μM, growth of both young and older roots was generally inhibited (22 h, $p = 0.04$; 26 h, $p < 0.0001$; and 30 h, $p = 0.01$; Fig. 4a).

ACC concentration at 0.01 μM did not appreciably affect the gravitropic curvature of 22 h roots, although it was reduced by 7–11% at higher concentrations of 1 and 50 μM; in comparison, ACC concentrations between 0.01 and 50 μM decreased the gravitropic curvature of 30 h roots by 7–19% (Fig. 4b). However, none of these effects of ACC on gravitropic curvature were statistically significant compared to the control treatment without ACC.

Ethylene Synthesis or Action Inhibitor Promotes Gravitropic Curvature of Older Roots

AVG, an inhibitor of ethylene synthesis, produced opposite effects on growth of young and old roots. In general, AVG inhibited growth of young roots (22 h) but stimulated growth of older roots (30 h) ($p \leq 0.05$; Fig. 5a). On the other hand, AVG stimulated gravitropic curvature of 30 h roots significantly by 24–26% at concentrations between 1 and 10 μM ($p = 0.03$ and 0.02); in contrast, AVG had no effect on curvature of 22- or 26-h roots (Fig. 5b).

The ethylene action inhibitor Ag^+ had no effect on growth of 22- or 30-h roots, although it inhibited growth of 26 h roots ($p < 0.05$; Fig. 6a). Similar to the ethylene synthesis inhibitor AVG, the ethylene action inhibitor Ag^+ increased gravitropic curvature of 30 h roots by nearly 32% at 10 μM Ag^+ ($p < 0.05$), but it produced no significant effect on curvature of 22- or 26-h roots (Fig. 6b).

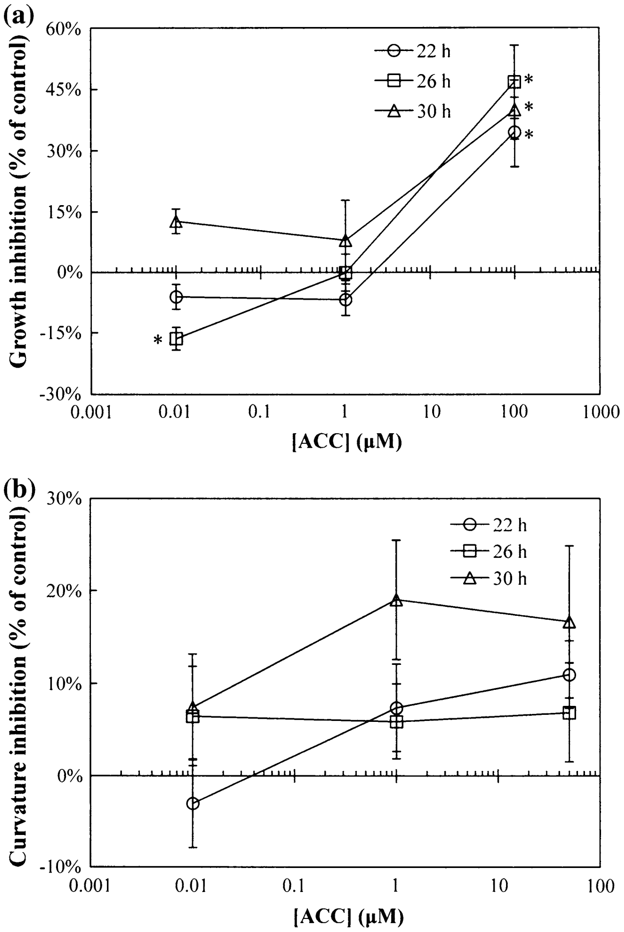

Fig. 4 The effect of the ethylene precursor ACC on **a** elongation and **b** gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. **a** Growth inhibition was calculated as the percentage reduction of growth rate compared to that of the control (that is, without ACC treatment) in each age group. Negative values in inhibition mean stimulation. Each data point represents a mean ± SE of more than 36 roots from three replicates. **b** Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature inhibition was calculated as the percentage reduction in curvature compared to that of the control (that is, without ACC treatment) in each age group. Negative values in inhibition mean stimulation. Each data point represents a mean ± SE of 27–72 roots from three replicates. In both **a** and **b**, statistical differences compared to the untreated roots at each age were determined by Student's *t* test; $*p \leq 0.05$

IAA Transport Inhibitor Decreases Gravitropic Curvature of Older Roots

Germination in the presence of NPA, an inhibitor of IAA transport, produced an inhibitory effect on growth of 22 h roots at 50 μM ($p = 0.008$) and of 30 h roots at 1 μM ($p = 0.01$), but it had no effect on growth of 26 h roots (Fig. 7a). NPA concentrations in the range of 1–50 μM stimulated gravitropic curvature of 22 h roots by 7–15%, although the effect was not statistically significant compared to that of the control treatment (that is, without NPA). In

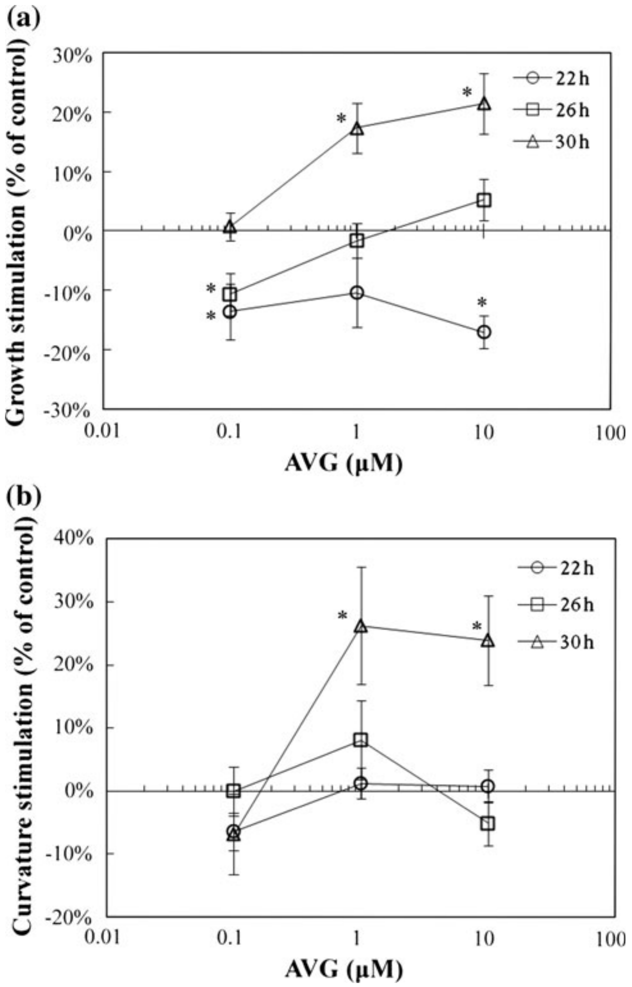

Fig. 5 The effect of the ethylene synthesis inhibitor AVG on **a** elongation and **b** gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. **a** Growth stimulation was calculated as the percentage increase in growth rate compared to that of the control (that is, without AVG treatment) in each age group. Negative values in stimulation mean inhibition. Each data point represents a mean ± SE of 30–60 roots from three replicates. **b** Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature stimulation was calculated as the percentage increase in curvature compared to that of the control (that is, without AVG treatment) in each age group. Negative values in stimulation mean inhibition. Each data point represents a mean ± SE of more than 42 roots from three replicates. In both **a** and **b**, statistical differences compared to the untreated roots at each age were determined by Student's *t* test; *$p \leq 0.05$

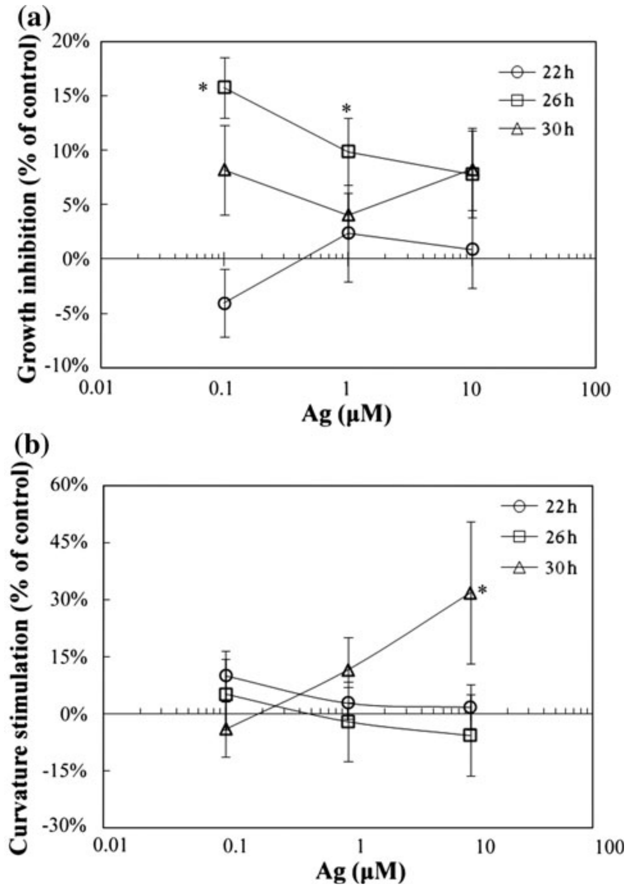

Fig. 6 The effect of the ethylene action inhibitor Ag^+ on **a** elongation and **b** gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. **a** Growth inhibition was calculated as the percentage reduction of growth rate compared to that of the control (that is, without Ag^+ treatment) in each age group. Negative values in inhibition mean stimulation. Each data point represents a mean ± SE of more than 24 roots from three replicates. **b** Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature stimulation was calculated as the percentage increase in curvature compared to that of the control (that is, without Ag^+ treatment) in each age group. Negative values in stimulation mean inhibition. Each data point represents a mean ± SE of more than 24 roots from three replicates. In both **a** and **b**, statistical differences compared to the untreated roots at each age were determined by Student's *t* test; *$p < 0.05$

contrast, NPA inhibited gravitropic curvature of 30 h roots by 21–24% ($p = 0.05$ at NPA = 50 µM) (Fig. 7b).

The inhibition of gravitropic curvature of older roots (30 h) due to the IAA transport inhibitor can be rescued by the addition of the ethylene synthesis inhibitor AVG or its action inhibitor Ag^+. When either AVG or Ag^+ was present in addition to NPA, the gravitropic curvature of older roots (30 h) was stimulated by 27–31% ($p = 0.001$ for AVG and 0.01 for Ag^+; Fig. 8). The same effect was not seen on younger (22- or 26-h) roots. However, the presence of 0.01 µM ACC along with NPA or 10 µM Ag^+ in the presence of 0.1 µM IAA both caused inhibition of gravitropic curvature of 30 h roots ($p = 0.01$ and 0.02, respectively; Fig. 9) without affecting younger roots (22 or 26 h).

Discussion

In our previous study (Ma and Hasenstein 2006), we found that gravisensitivity is initially established in flax embryonic roots during seed imbibition, prior to germination.

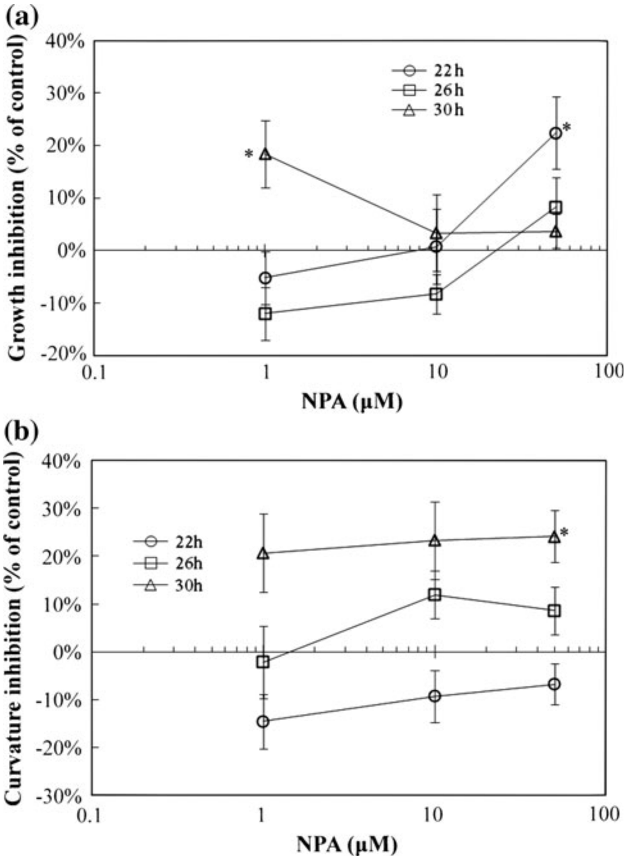

Fig. 7 The effect of the auxin transport inhibitor NPA on **a** elongation and **b** gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. **a** Growth inhibition was calculated as the percentage reduction of growth rate compared to that of the control (that is, without NPA treatment) in each age group. Negative values in inhibition mean stimulation. Each data point represents a mean ± SE of more than 36 roots from three replicates. **b** Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature inhibition was calculated as the percentage reduction in curvature compared to that of the control (that is, without NPA treatment) in each age group. Negative values in inhibition mean stimulation. Each data point represents a mean ± SE of 21–48 roots from three replicates. In both **a** and **b**, statistical differences compared to the untreated roots at each age were determined by Student's *t* test; $*p \leq 0.05$

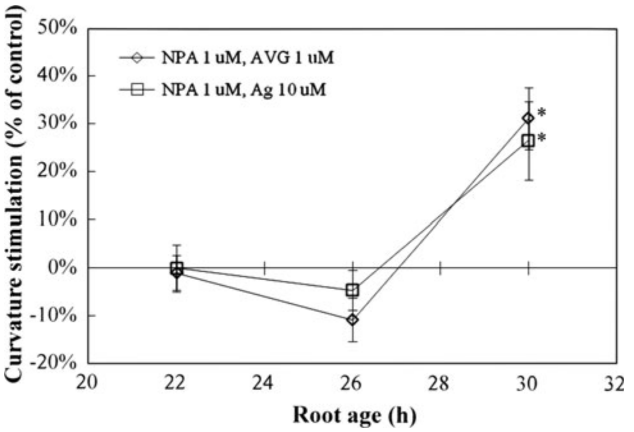

Fig. 8 The effect of the auxin transport inhibitor NPA, along with either the ethylene synthesis inhibitor AVG or the ethylene action inhibitor Ag^+, on gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature stimulation was calculated as the percentage increase in curvature compared to that of the control (that is, without NPA, AVG, or Ag^+ treatment) in each age group. Each data point represents a mean ± SE of 30–78 roots from three replicates. Statistical differences compared to the untreated roots at each age were determined by Student's *t* test; $*p \leq 0.01$

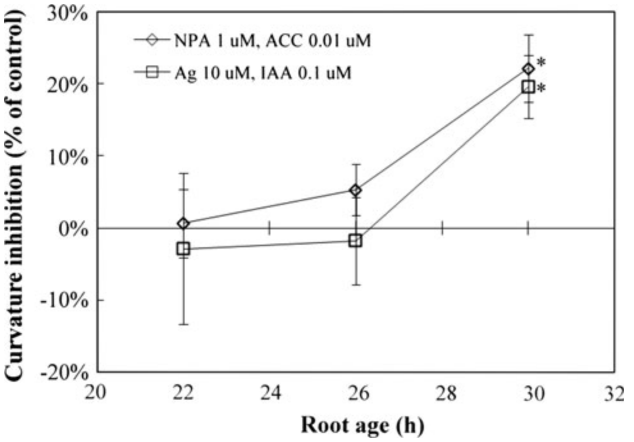

Fig. 9 The effect of the auxin transport inhibitor NPA along with the ethylene precursor ACC, and the effect of the ethylene action inhibitor Ag^+ along with IAA on gravitropic curvature of roots of three different ages, that is, 22, 26, or 30 h. Root curvature was measured on roots that were gravistimulated horizontally for 3 h. Curvature inhibition was calculated as the percentage reduction in curvature compared to that of the control (that is, without NPA, ACC, Ag^+, or IAA treatment) in each age group. Each data point represents a mean ± SE of 24–51 roots from three replicates. Statistical differences compared to the untreated roots at each age were determined by Student's *t* test; $*p \leq 0.02$

Subsequently, we observed that the gravity response of flax (and corn) roots weakened over time after root emergence, before seedling shoot emergence. In flax roots, the gravity response deteriorated rapidly from 3 to 11 h after root emergence (Fig. 1). It is interesting that gravitropism is strongest in newly emerged roots but then becomes attenuated over time as roots grow. Such developmental modification to gravitropism could indicate changes in the gravity-sensing machinery and/or the response mechanism.

The gravitropic curving rate of flax seedling roots decreased continuously as roots aged from 22 to 30 h. During a 4 h period of horizontal gravistimulation, the curving rate of 26- and 30-h roots dropped by 44 and 60%, respectively, compared to that of 22 h roots (Fig. 2). On the other hand, the vertical root elongation rate of 26- and 30-h roots increased by 32 and 15%, respectively (Fig. 2). Changes in root growth rate likely reflect fluctuations in auxin status in the elongation zone, which in turn may affect root curving. However, the formation of gravitropic curvature results from differential growth induced by an auxin gradient between the upper and lower flanks of the root. Therefore, the dramatic decrease in the gravitropic curving rate of older roots suggests a reduction in the magnitude of auxin differential and/or in tissue sensitivity to auxin as a result of changes in auxin synthesis, conjugation, and/or transport.

Young (that is, 22 h) and relatively old (that is, 26- and 30-h) roots responded differently in both elongation and gravitropic response when incubated with exogenous IAA. Growth of 22 h roots was inhibited at a much lower IAA concentration (estimated at >0.2 nM) compared to that of 30 h roots (estimated at >110 nM), a tolerance difference of nearly 600 times (Fig. 3a). This indicates that tissues are more sensitive to IAA in younger rather than older roots, which is in agreement with changes in tissue sensitivity to auxin caused by aging or in response to gravistimulation reported previously in other studies (Rorabaugh and Salisbury 1989; Salisbury and others 1988; Went and Thimann 1937). On the other hand, gravitropism of the young roots (that is, 22 h) was inhibited three to four times more than that of the older roots (that is, 26- and 30-h) at a low exogenous IAA concentration (that is, 10 nM), although a higher IAA concentration (that is, 1,000 nM) inhibited gravitropism of both the young and the old roots equally (Fig. 3b). Because the smaller the endogenous auxin gradient across the root the more easily it would be disrupted by an exogenous background IAA bathing the root tissue, it can be argued that young roots are capable of responding to gravity in the presence of a smaller auxin gradient, whereas older roots would require a greater auxin gradient for a similar degree of response.

Decreased tissue sensitivity to auxin could lead to the attenuated gravitropic response of older roots. As root tissue becomes less sensitive (that is, more tolerant) to auxin with aging, as shown in our experiments (Fig. 3a), elongation of cells situated at the upper and lower flanks of a horizontally oriented root would increase or decrease only with more auxin present in order to set the stage for the differential growth required for curvature development. This can be achieved by having more auxin synthesis, release from auxin conjugates, and transport from cells at the root tip. Therefore, the development and extent of gravitropic curvature of old roots depends on whether or how quickly auxin synthesis, release from auxin conjugates, or transport can occur. An important and interesting question is: What causes the change in tissue sensitivity to auxin with root aging? We hypothesized that ethylene was involved in the process. As a plant hormone, ethylene affects various aspects of growth and development and is involved in many stress responses (Alonso and Stepanova 2004; Chen and others 2005; Lin and others 2009; Ma and others 2003; Negi and others 2008). Ethylene can regulate root growth by mediating biosynthesis and transport of auxin (Růžička and others 2007; Swarup and others 2007). Ethylene can also regulate lateral root formation along with auxin (Ivanchenko and others 2008; Negi and others 2008). In addition, ethylene acts as a modulator of root gravitropism (Abeles and others 1992; Buer and others 2006; Madlung and others 1999; Mattoo and Suttle 1991) and has been reported to inhibit the gravity response of *Arabidopsis* roots by altering the synthesis of flavonoids, principal candidates for endogenous auxin transport inhibitors (Buer and others 2006).

If ethylene acted to decrease tissue sensitivity to auxin or negatively impact on auxin synthesis or transport as roots age, then we would expect to see a weakened gravitropic response with root growth. We tested ethylene involvement by employing the precursor of ethylene synthesis (ACC), the ethylene synthesis inhibitor (AVG), and the ethylene action inhibitor (Ag^+), in combination with the auxin transport inhibitor (NPA). In all experiments, root elongation and/or gravitropism was affected, indicating that root uptake of these compounds was effective. The ethylene precursor ACC inhibited elongation at a high concentration of 100 µM, but it did not affect gravitropic curvature significantly (Fig. 4a, b). On the other hand, the inhibitor of ethylene synthesis (AVG) or action (Ag^+) produced varied effects on root elongation (Figs. 5a, 6a) but stimulated gravitropic curvature of older (that is, 30 h) roots by 24–32% without having any effects on young (that is, 22 h) roots (Figs. 5b, 6b). This suggests an age-dependent effect of ethylene on gravitropism and could partly account for inconsistent results of ethylene effects on gravitropism as reported in a number of earlier studies employing various plant materials of different ages (Buer and others 2006; Chang and others 2004; Kiss and others 1999; Lee and others 1990; Madlung and others 1999; Wheeler and others 1986). Together, these results suggest that ethylene plays a developmental role in root gravitropism. Because ethylene affects elongation and gravitropism differently (Figs. 4, 5, 6), this suggests that the effects are independent of each other, which is in agreement with earlier reports (Evans and others 1989; Hensel and Iversen 1980). Because gravitropism in young roots did not respond to ethylene synthesis or action inhibitors (Figs. 5b, 6b), it is likely that young roots have either a low level of endogenous ethylene or a low tissue sensitivity to this hormone. As roots age, ethylene synthesis and response could increase. This could explain why additional ACC did not induce further inhibition of gravitropic response of old roots (Fig. 4b) if the already high level of endogenous ethylene had led to significant inhibition. Aging-induced ethylene synthesis and response would be important for the development of more complex root system architecture originating from the primary root, considering that ethylene is involved in the crosstalk with auxin in regulating lateral root formation and auxin transport (Ivanchenko and others 2008; Negi and others 2008).

Our experiments demonstrate that ethylene is involved in the weakening of gravitropism of the flax primary root as it ages. Increased ethylene synthesis with root aging could desensitize cells to auxin, although it is not clear whether ethylene affected auxin synthesis or release from auxin conjugates. However, it is possible that ethylene inhibited auxin transport in an age-dependent manner. The auxin transport inhibitor slightly stimulated gravitropic curvature of 22 h roots but strongly inhibited gravitropic curvature of 30 h roots (Fig. 7b). This is not surprising, considering that young roots exhibit higher auxin sensitivity and hence may respond to a smaller auxin gradient to produce a gravitropic response (Fig. 3). With greater auxin sensitivity, cell elongation at the upper and lower flanks would be stimulated or inhibited at a lower auxin concentration, and it would respond to a reduced amount of auxin by displaying differential elongation. With aging-related decrease in auxin sensitivity, differential root growth would not occur as fast until the auxin concentration increased beyond a higher threshold level (Fig. 3a). As a result, reduced auxin transport in older roots would be detrimental to establishing the larger auxin gradient needed for differential elongation, leading to inhibited gravitropic curvature (Fig. 7b). However, this reduced gravitropism of older roots due to the presence of the auxin transport inhibitor was reversed by either ethylene synthesis or its action inhibitor (Fig. 8), suggesting that ethylene could have inhibited auxin transport in older roots. This is consistent with earlier studies showing that polar transport and lateral redistribution of IAA is inhibited by ethylene (Burg and Burg 1966; Lee and others 1990; Morgan and Gausman 1966; Prayitno and others 2006; Suttle 1988) and that reduction in IAA transport occurred with advancing tissue age (Davenport and others 1980; Suttle 1991). Although ACC combined with NPA did not inhibit gravitropism of older roots more than NPA did by itself (Fig. 9), this could indicate a saturation effect of both endogenous ethylene and NPA on auxin transport. On the other hand, the ethylene action inhibitor Ag^+ combined with exogenously supplied IAA reduced gravitropism of older roots, likely resulting from a disrupted endogenous auxin gradient by external IAA (Fig. 9).

In summary, our data support a role for ethylene in regulating the developmental attenuation of root gravitropism of flax by affecting auxin sensitivity and possibly its transport (Fig. 10). In addition to this growth-related weakening in the gravitropic response, we have also detected prolonged presentation time in old roots (data not shown), an indication of developmental modification to the gravity-sensing machinery itself. Future studies will examine developmental changes in the structure of the root cap (for example, number and size of amyloplasts, actin network, and so on) in relation to auxin and ethylene. These studies are expected to yield new insights into the temporal dynamics of the development of the root gravity-sensing machinery.

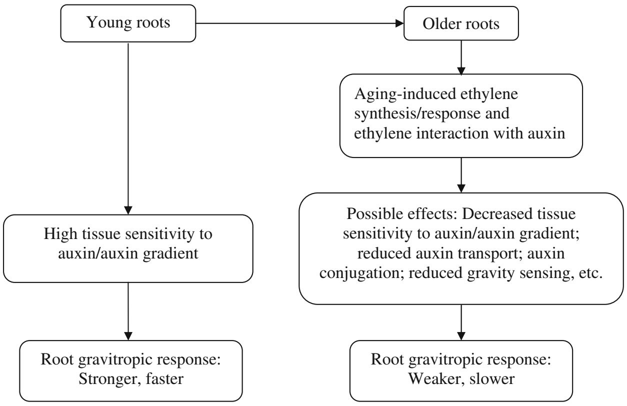

Fig. 10 A model for the involvement of ethylene in the developmental attenuation of root gravitropism

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